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THE BIOLOGICAL ACTION OF THYROXINE ON EMBRYONIC BONES GROWN IN TISSUE CULTURE

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Tissue culture technique has proved to be of value in the study of the biological action of vitamin A (Fell & Mellanby, 1952, 1953), and it seemed possible that it might be useful in investigating the direct action on tissue of other biological substances such as the active principles of the thyroid gland. Many previous investigations into the action of thyroid active substances by tissue culture techniques have been reported, but it has proved to be an unprofitable field. The subject has been reviewed by Barker (1951) and he sums up the position as follows: 'A conservative summary of the present status of this aspect of thyroid function would be that no *in vitro* response to thyroxine or thyroglobulin has yet been consistent enough to furnish a means of probing into the detailed mechanism of the action of the hormone.' Many efforts have, for instance, been made to see what thyroxine does to the growth of fibroblasts *in vitro*, but the wide variation in results only emphasizes the general conclusion of Barker (Semura, 1931; Vogelaar & Erlichman, 1936; Ebeling, 1924; Verne & Odiette, 1936; Latta & Davis, 1938; van Hamm & Cappel, 1940).

Embryonic chick bones were chosen as the biological material for the present work largely because of previous evidence that the thyroid gland is involved in bone growth and maintenance of structure, since both deficiency and excess of its active principles produce bone abnormality. Clinical evidence based on radiographic examination from many quarters has shown that osteoporosis is common in hyperthyroidism (Kummer, 1917; Bernard, 1927; Plummer, 1928; Aub, Bauer, Heath & Ropes, 1929; Hunter, 1930). Heath, Bauer & Aub (1926) and Aub *et al.* (1929) found that both calcium and phosphorus excretion was greatly increased in hyperthyroidic patients, suggesting that these substances came from the calcium phosphate of the bones. In myxoedema, on the contrary, the calcium excretion was below that of normal individuals. Hunter (1930) reported two cases of hyperthyroidism with spontaneous fractures in

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which there was radiographic evidence of decalcification in all bones examined as well as in the fractured bones. He also found that the osteoporosis was brought about by osteoclastic lacunar absorption which affected not only the corticalis but also the spongiosa of the medullary cavity, and he stated that there was no cessation of calcification as in rickets and osteomalacia. It is of interest in this connexion that one of us had found previously that dried thyroid gland added to the diet of puppies deficient in vitamin D, increased the development of a condition which radiographically was indistinguishable from rickets (Mellanby, 1923). On the other hand, Smith & McLean (1938) were unable to observe any failure of calcification in the bones of rapidly growing rats in severe hyperthyroidism.

The bones of cretins are well known to show great interference with growth and development. In a cretin aged 15 Eaves & Croll (1928) described an unusually large amount of unchanged cartilage in the femur and ribs. At the end of a femur of this patient, the unchanged cartilage was several inches in length. They also found that the cartilage cells in a rib were very irregular and the ossification process erratic. It is therefore clear that clinical evidence shows that in both hyper- and hypothyroidism in man, bone is greatly affected, and it seems probable that variations in the amount of thyroid-active principles in these conditions is responsible either directly or indirectly for the different bone changes described.

There is also evidence from laboratory experiments that skeletal growth is influenced by thyroxine. Thus Ray, Asling, Simpson & Evans (1950) found that the injection into hypophysectomized rats of 3 μg thyroxine daily from the 30th to the 60th day of life prevented the arrest of endochondral ossification which occurred in the control hypophysectomized animals. In the thyroid-injected rats erosion of cartilage and replacement by bone continued, although chondrogenesis itself was not maintained. Retardation and acceleration of epiphyseal activity in kittens and puppies in athyroidism and hyperthyroidism respectively were described by Dott (1923). In experimental hyperthyroidism he found in the cartilage an indication of increased rate of cell proliferation and in the matrix signs of rapidly increasing maturity; although growth was accelerated, maturity also was disproportionately hastened, so that stature became fixed at a subnormal limit by epiphyseal ossification. Silberberg & Silberberg (1938, 1940) later found that dried thyroid given by mouth to young guinea-pigs and the injection of thyroxine into young mice accelerated and intensified the age changes in the skeleton. Simpson, Asling & Evans (1950) also found that injection of thyroxine into the young rat and mouse caused premature ageing of the skeleton as well as acceleration of the differentiation of the long bones. Thyroxine alone had no growth-promoting effect, but increased that of the growth hormone when both were administered together.

It is well known that in the amphibia thyroxine accelerates metamorphosis but retards growth (Gudernatsch, 1912, 1917). Some work has been done on the effect of thyroxine on the development of chick embryos *in ovo*, but the results of these experiments were conflicting. Willier (1924) grafted fragments of thyroid from hatched chicks on to the chorio-allantoic membranes of 7 to 10-day embryos. When examined on the 17th day of incubation, the treated embryos were found to be considerably smaller than the controls, and there was 'shortening and emaciation of the segments of the wings and particularly of the legs'. Two investigators have injected thyroxine into either the embryo or the egg. Guelin-Schedrina (1933) gave one to five injections of synthetic thyroxine (0·01–0·001 mg) into the heart or blood vessels of 3-day embryos, but noted only non-specific toxic effects. Beyer (1952), on the other hand, injected thyroxine into eggs before incubation and found that a dose of 0·025 μg caused an increase in both the dry and wet weights of the embryos and in their total oxygen consumption, but not in their metabolic rate per g weight. The disparities between the results of these three workers are probably due to the widely different conditions of their experiments, but this point will be discussed later.

The present study was made to see whether thyroxine, in concentrations similar to those which can be found in the living animal, would affect skeletal rudiments isolated *in vitro*. The results have shown that the hormone has a direct action under these conditions, that its effect depends on the degree of differentiation of the skeletal tissue at the beginning of the experiment and that the various limb-bone rudiments react differently to the same dose.

MATERIAL AND METHODS

Materials

Skeletal explants were obtained from the leg- and wing-buds of 4 to 7-day embryonic chicks. As there was considerable individual and seasonal variation in the development of embryos of the same age, the limb-buds were classified in four groups according to their stage of differentiation. In group I (14 chicks) there was usually a mesodermal condensation in the leg-buds but in the wing-buds the condensation was less distinct; in group II (8 chicks), both buds contained a continuous pro-cartilaginous blastema; in group III (9 chicks), individual bone rudiments were distinguishable but chondroblastic hypertrophy had not yet begun in the shafts; in group IV (11 chicks), hypertrophy had appeared in one or more bone primordia. In groups III and IV the rudiments of an additional 76 embryo chicks were cultivated for purposes of measurement and general structure, but they were not examined histologically.

In groups I and II, the skeletogenous core was dissected from the buds and explanted whole, but in groups III and IV, where joints were already present, the individual primordia were cultivated separately. In all, 104 pairs of skeletal explants were grown and examined histologically; of each pair, one rudiment was cultivated in medium containing thyroxine and the other in normal medium to serve as a control.

Methods

Tissue culture. The rudiments were grown by the watch-glass method which has already been described elsewhere (Fell & Robison, 1929; Fell & Mellanby, 1952). The culture medium consisted

of 3 parts plasma: 1 part embryo extract; at the beginning of the experiment, 9 drops of plasma + 3 drops of embryo extract were placed in each watch-glass, but as the explants enlarged, the amount was raised to 12 drops and 4 drops respectively.

The embryo extract was made from 13 to 14-day embryos, very finely minced and ground. Equal parts of mince and Tyrode containing 1% (w/v) glucose were mixed and centrifuged for not more than 5 min at about 2000 rev/min. With some of the mince a more dilute extract was prepared with Tyrode containing the usual amount of glucose (0·1%); the explants were placed in a drop of this extract before being transferred to the clot in the watch-glass, so that, when the surplus fluid was removed with a fine pipette, the tissue was covered by a film of growth-promoting liquid. The explants were transplanted at 2-day intervals.

Addition of thyroxine to plasma. Most of the experiments here described were done with plasma designated X_2. This contained, in addition to its normal content of thyroxine, an added quantity which in the culture medium amounted to approximately 16 μg/100 ml. of medium. It was prepared as follows: a solution of L-thyroxine in a sterile 0·1% (w/v) solution of sodium carbonate was made by dissolving 1 mg in 32 ml. (solution A). Solution A was sterilized by boiling. To each 4·5 ml. of bird plasma 0·03 ml. of solution A were added, i.e. 100 ml. of plasma now contained 20·8 μg of added L-thyroxine. To make the medium for the experiments, 3 parts of plasma were added to 1 part of embryo extract, so that the final concentration of added L-thyroxine in the medium was approximately 16 μg/100 ml. of medium in addition to the normal content of thyroxine and triiodothyronine. In some experiments a culture medium described as X_6 has been used; 1 ml. of solution A was added to 15 ml. of 0·1% sterile sodium carbonate solution and the same procedure was followed as above, so that X_6 medium contained 1 μg of added L-thyroxine per 100 ml. of culture medium. The same amount of Na_2CO_3 was added to the control medium in each experiment.

Measurement. Every 2 days the explants were drawn with the aid of a camera lucida, at a known magnification. The drawings were measured with a piece of string infiltrated with paraffin wax which served as a flexible ruler; the final result was expressed in mm. In another group of experiments the rudiments were measured directly with a micrometer eye-piece.

The measurements of the bones which formed the data for the curves shown in Text-figs. 5–7 were submitted to Dr J. W. Boag for statistical examination. By tabulating the growth increments in successive time intervals he obtained the mean value for δ (the difference in amount of growth between the thyroxine-treated and the control bones) in each series of experiments. He then applied the usual statistical test (Student's t-test) to see whether the mean value of δ differed significantly from zero. Where differences of statistical significance were obtained by this method, this is mentioned in the text.

Histology. The rudiments were fixed at various intervals in 3% acetic Zenker's solution for about 30 min. After being embedded in paraffin wax, they were serially sectioned. Slides were stained with Delafield's haematoxylin and chromotrop, azan, toluidine blue or Mayer's acid haemalum and chromotrop.

RESULTS

The normal development and growth of the long-bones in the embryonic chick

The histogenesis of cartilage and bone in the long-bone of the embryonic fowl has been described in some detail by one of us (Fell, 1925), but this earlier work did not include a comparative study of differentiation in the various rudiments; recent observations have shown that there is some variation in the histogenetic process in the different bones.

The general course of differentiation is the same in all the primordia. At about the 6th day the cells in the middle segment of the shaft begin to enlarge, while on either side of this region a broad zone of flattened cells merges

terminally with the small-celled epiphyses; the articular surfaces are not yet sharply defined. Over and slightly beyond the hypertrophic region, the perichondrium has differentiated into a periosteum with an inner osteoblastic and an outer fibroblastic coat, and a delicate layer of intercellular fibres has usually been deposited between the oesteoblasts and the cartilage.

About 24 hr later, many of the enlarged cells have assumed their characteristic rounded shape and swollen vacuolated appearance. This change is always most advanced in the interior of the shaft, i.e. in the first formed chondroblasts, whence it spreads peripherally and distally. The zones of flattened cells are becoming demarcated from the epiphyses, the articular surfaces are now distinct, the periosteum extends almost to the ends of the shaft and a simple layer of bone has been formed. The hypertrophic region continues to enlarge and the sheath of periosteal bone to thicken. By the 8th day, the osteoblastic layer has become vascularized and, in addition to the original sheath of bone, trabeculae are being formed between the vessels.

Between the 10th and 12th day, blood vessels and connective tissue invade the diaphysial cartilage which is excavated to form the marrow-cavity. In the chick there is no endochondral ossification until the later stages of development. As the cartilage is scooped away from the middle segment of the shaft and replaced by marrow, more hypertrophic cartilage is formed distally from the zone of flattened cells, while the bone of the diaphysis becomes steadily thicker and better developed.

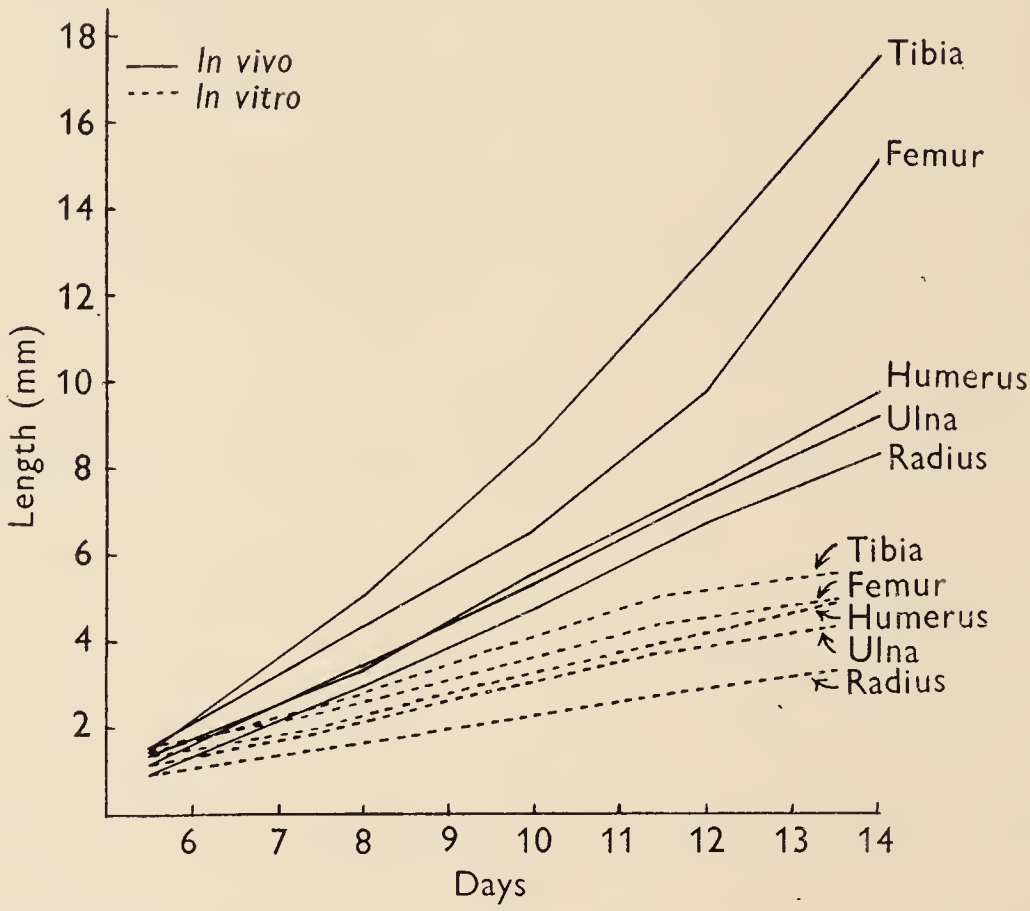
When the individual rudiments are examined, it is found that at about the 6th day hypertrophy of the cartilage cells is most advanced in the femur, then in the humerus, next in the tibia and ulna, but it has not yet appeared in the radius. In a 7-day embryo, the degree of hypertrophy follows the same sequence, but camera lucida drawings show that the hypertrophic region has enlarged much more in the tibia than in the femur or humerus; thus while at the 6th day the tibial hypertrophic zone is smaller than that of the femur or humerus, by the 7th day it is nearly twice as large. The hypertrophic area of the ulna has also enlarged considerably and one has now appeared in the radius.

Up to the 10th day the hypertrophic zone continues to extend more rapidly in the tibia than in the other rudiments, although the degree of hypertrophy remains most advanced in the femur. The radius differentiates more slowly than any of the bones and hypertrophy never reaches such an advanced stage as in the other rudiments. The hypertrophic cartilage of the ulna is better developed than that of the radius but remains inferior to that of the humerus and the leg-bones.

After 12–16 days, when the long-bones have acquired a marrow cavity, histological differences between the various bones are still seen. In the tibia and, to a lesser degree, in the femur also, the cells of the hypertrophic cartilage,

especially those towards the periphery of the shaft, are rather larger and set more closely together than in the humerus. The hypertrophic chondroblasts of the ulna, and especially of the radius, are less swollen than in either the humerus or the leg-bones and are separated by broader partitions of matrix.

Thus it is seen that, although all the long-bones develop according to the same general plan, their histogenesis *in vivo* is not identical. There are also differences between the normal growth-rates of the various rudiments. It will be seen from Text-fig. 1 that the tibia is the most rapidly growing bone, next the femur and then in order the humerus, ulna and radius.



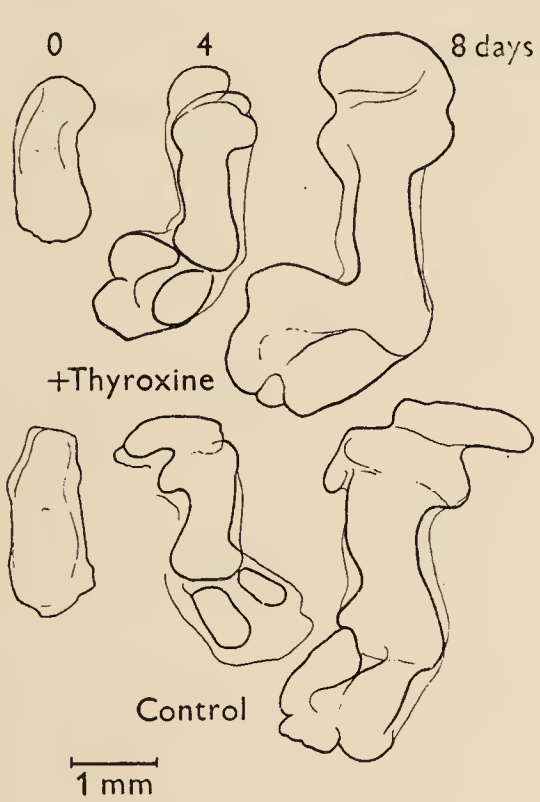
Text-fig. 1. Average growth curves of the long-bones of the embryonic chick. Note the great differences in the growth of the bones *in vivo* (plain lines), the tibia being the most rapidly growing and the radius the least. *In vitro* (dotted lines) the growth of the bones is much slower and the differences are much smaller, but the tibia and radius retain their relative positions. Ordinates represent length of bones in mm; abscissae represent days of incubation or (*in vitro*) of culture.

The effect of thyroxine on explanted rudiments of groups I and II

Development. In the original explants, individual primordia were indistinguishable in the living blastema which appeared as a dense, oblong mass surrounded by rather more translucent myogenic tissue. The gross anatomical development of the explants was the same in both the thyroxine-treated (T-treated) and control cultures, and the following description applies to both.

After 2 days' growth, the various bone-rudiments were recognizable, though still indistinct in group I, and by the 4th day were very clearly seen in all cultures. At this stage the wing-skeleton (Text-fig. 2) consisted of a humerus, usually associated with fragments of the pectoral girdle, a radius, ulna, often

part of the carpus and in group II sometimes pieces of the metacarpus: in the younger members of group I, the distal ends of the radius and ulna were sometimes missing. The leg-skeleton at the 4th day comprised a femur, often with part of the pelvis attached, a tibia, fibula, tarsus and sometimes incomplete metatarsal elements (Text-fig. 3). In both the leg- and wing-skeleton, joints developed at the knee, elbow, shoulder and hip.



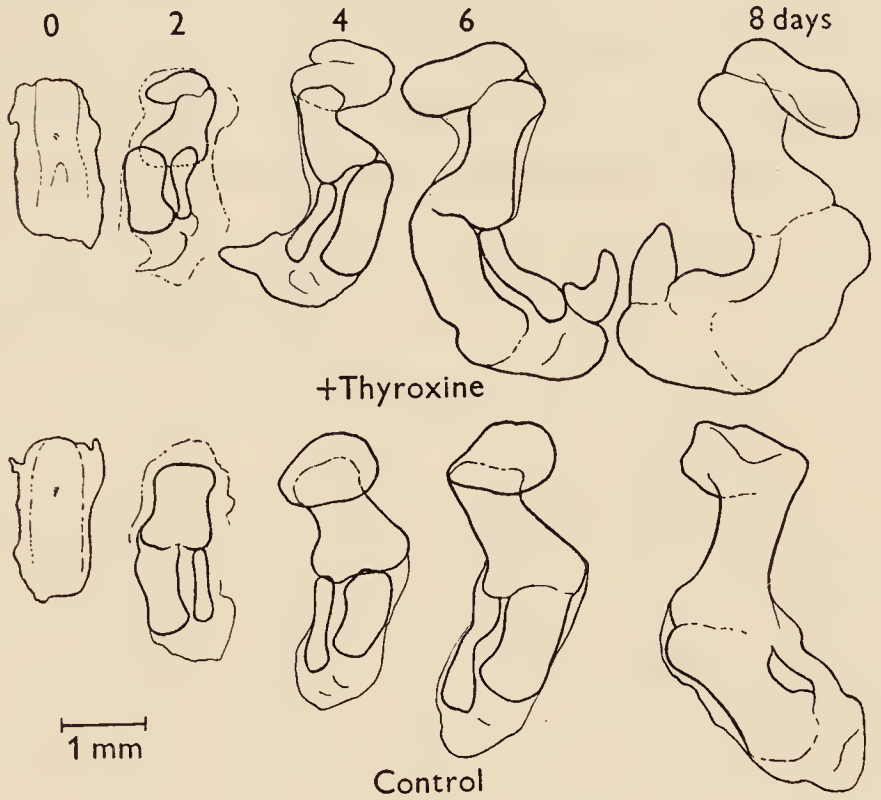


Fig. 2 Fig. 3

Text-fig. 2. Camera lucida drawings of the living skeletal blastemata from the wing-buds of an embryo of group II; one was grown in medium containing added 16 μg thyroxine/100 ml., the other in control medium. The humerus is considerably longer in the T-treated explant and in section was found to be much more highly differentiated than the corresponding rudiment in the control. Note the poor development of the radius and ulna in both explants.

Text-fig. 3. Camera lucida drawings of the living skeletal blastemata from the leg-buds of an embryo of group I; one was grown for 8 days in medium containing 16 μg thyroxine/100 ml., the other in control medium. Note the anatomical development that has taken place in both explants; by the 8th day the joints are much less distinct owing to fusion of the articular surfaces. The tibia in the T-treated explant has undergone hypertrophy and ossification and is longer than that in the control.

After the 4th day, the joints gradually became less distinct owing to secondary fusion of the articular surfaces, and at the same time the relative proportions of the rudiments usually changed. In the wing, the humerus sometimes grew to several times the length of the radius and ulna, but in the leg there was less disproportion and the femur and tibia usually remained about equal in length; if metatarsal elements were present, however, they grew very rapidly in relation to the rest of the skeleton.

The greatest differences in growth rate occurred when one of the rudiments underwent chondroblastic hypertrophy and another did not. The hypertrophic cartilage, which was often recognizable in the living cultures at the 4th day,

appeared by transmitted light as a well-defined, rather yellowish area in the middle of the shaft, and its differentiation was always associated with a rapid expansion of the cartilage. In some rudiments the hypertrophic cartilage extended right across the shaft in the normal way, but in others, especially in group I, it appeared on one side only and there produced a bulge or nose-like projection due to the asymmetrical expansion of the shaft.

It could be seen from the living cultures that the incidence, extent and often the degree of chondroblastic hypertrophy were much greater in the T-treated explants than in the controls. This was confirmed by histological examination, and indicated that the hormone had stimulated differentiation. Table 1 shows

TABLE 1. Groups I and II

		No. of rudiments with hypertrophic cartilage											
		Wing							Leg				
	Added thyroxine (μg/100 ml)	No. of pairs of explants	Humerus		Radius		Ulna		No. of pairs of explants	Femur		Tibia	
			T	*C*	*T*	*C*	*T*	*C*		*T*	*C*	*T*	*C*
Group I	1	5	5	3	0	0	0	0	5	1	1	4	0
	16	9	9	2	0	0	0	0	9	1	0	3	0
	Total	14	14	5	0	0	0	0	14	2	1	7	0
Group II	1	2	2	1	0	0	0	0	2	1	0	2	1
	16	6	6	5	3	0	0	0	6	4	1	4	3
	Total	8	8	6	3	0	0	0	8	5	1	6	4
Group I + Group II	Total	22	22	11	3	0	0	0	22	7	2	13	4

Incidence of hypertrophic cartilage in the long-bone rudiments of paired blastemata explanted from 4 to 5-day wing- and leg-buds; 8–9 days' cultivation *in vitro*. *T* = *medium + thyroxine*, *C* = control medium; one of each pair was grown in *T* and the other in *C*. For definition of groups see p. 429.

that in control medium the incidence of hypertrophy was greater in certain rudiments than in others, and in general was higher in group II than in group I. It will also be seen that both concentrations of thyroxine had a stimulatory effect, and that those rudiments in which hypertrophy was most frequent in control medium were the most readily stimulated by the hormone. Thus, listing the rudiments of the 22 blastemata in order of the frequency with which they underwent hypertrophy in control medium, we find that hypertrophic cartilage was present in 11 of the 22 control humeri but in all 22 of those treated with thyroxine (Pl. 1, figs. 1, 2), in 4 of the 22 control and 13 of the T-treated tibiae, in 2 control and 7 T-treated femora, in none of the control and in 3 T-treated radii, and in none of either the control or T-treated ulnae.

The general histological structure of the explants was the same in both the T-treated and control series. The hypertrophic cartilage had the characteristic round, vacuolated cells, and was separated from the small-celled epiphyses by zones of flattened cells as in normal development; it was covered by a 2-layered

periosteum and a sheath of bone had been deposited on the surface. In other rudiments there were areas of slightly enlarged cells which may have represented the first stage in hypertrophy, but which are not included in Table 1. Those primordia which did not ossify were composed of small-celled cartilage which appeared healthy and had abundant matrix.

As stated above, in 11 pairs of humeri hypertrophic cartilage was present in both the T-treated and control members. In 10 of these pairs, hypertrophy was more advanced and extensive in the T-treated rudiment than in the control, and in the 11th the degree of differentiation was the same in both. In the other rudiments in which both members of a pair had undergone hypertrophy, there was either no difference between the treated and untreated primordia (femora and 1 pair of tibiae), or the T-treated rudiment was more advanced (2 pairs of tibiae). Only one T-treated rudiment was less developed than its control; this was a tibia which was much distorted, probably owing to damage during dissection.

Two of the humeri of group II exposed to the higher concentration of thyroxine showed regressive changes of a type which, though rare in group II, was often seen in the T-treated rudiments of groups III and IV (see below). In these two humeri many of the hypertrophic cells had retracted from the capsular wall; the space between the cytoplasm and the wall was occupied by a loose network of matrix which in the final stages of degeneration completely filled the cavity, the chondroblast being reduced to a small, shrunken mass in the centre. At the same time the partitions between the peripheral chondroblasts, i.e. those most recently hypertrophied, were abnormally thin; this effect will be considered in more detail later.

Summarizing these results, we find that in the 22 pairs of leg- and 22 pairs of wing-blastemata, thyroxine stimulated differentiation in 21 humeri, 15 tibiae, 7 femora, 3 radii and no ulna; in only one treated rudiment was differentiation inferior to that of its control.

Growth. Owing to the partial obliteration of the articular lines and the frequent curvature and overlap of the distal rudiments during the later stages of cultivation, it was only possible to measure the growth rates of the humerus and femur, which usually remained sufficiently straight and in which the articular boundaries were still distinguishable after fusion of the opposed surfaces.

Text-fig. 5 shows the relative growth rate (*a*, *b*) and the average growth curves (*c*, *d*) of 12 pairs of humeri and 10 pairs of femora, in which the T-treated explants were grown in medium containing 16 μg/100 ml. of thyroxine. Since the individual rudiments had not differentiated in the blastemata at the time of explantation, measurement began on the 2nd day in culture. It will be seen that between the 2nd and 4th days, the humeri grew more rapidly in the presence of thyroxine than in the control medium; this result was found to be

statistically significant. The growth rate then declined relatively to that of control humeri until it fell below that of the controls. The T-treated femora, on the other hand, grew at the same rate as their controls during the first 6 days, after which there was a sudden drop in the growth rate (statistically significant) and the thyroxine curve fell below that of the control.

As described above, chondroblastic hypertrophy always causes a rapid expansion of the cartilage in which it occurs, and it is probable that the sharp rise in the rate of elongation of the T-treated humeri between the 2nd and 4th day was associated with the marked stimulation of hypertrophy produced by thyroxine in all the twelve rudiments measured. Of the ten T-treated femora from which the average growth curve was obtained, hypertrophy was present in only two. The fact that in the presence of thyroxine the growth rate of both the humeri and femora fell below that of the controls after the 6th day, suggested that more prolonged exposure to the hormone was harmful. This was supported by the observation that by the 8th day of cultivation the zone of outgrowth from the connective tissue surrounding the skeleton was sparse and unhealthy in contrast to its appearance in the controls.

The effect of thyroxine on explanted rudiments of groups III and IV

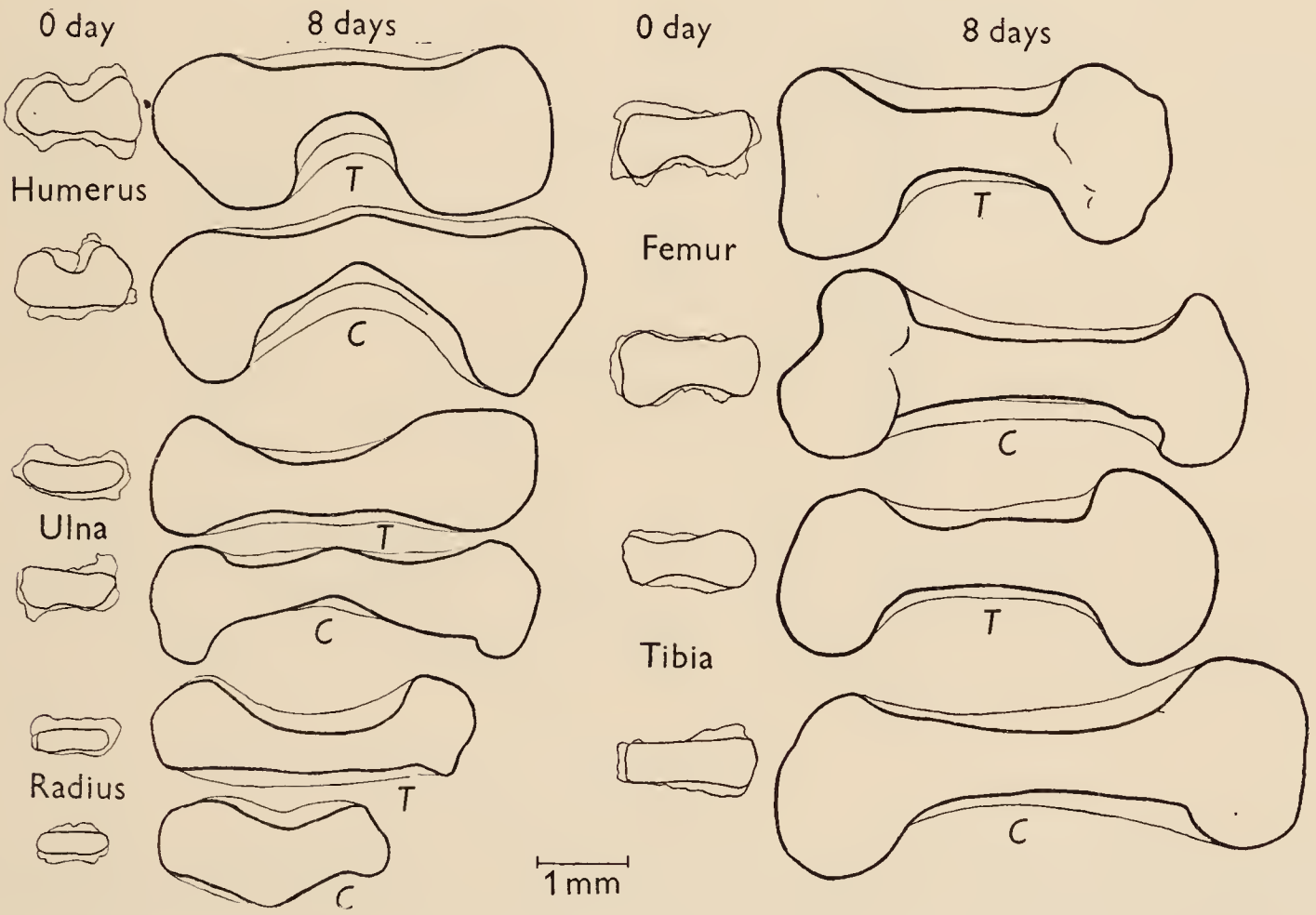
Development. When first removed from the embryo, the long-bone rudiments of group III (Text-fig. 4) were short and stumpy and showed little anatomical or histological differentiation; those of group IV were better developed and had begun to assume their characteristic shapes, while with transmitted light yellowish areas of hypertrophic cartilage were seen in the femur, humerus and sometimes in the other primordia.

When grown in control medium, the humerus in group III formed its head and trochanter, the distal end acquired a fairly normal outline and the shaft elongated rapidly and became curved as *in vivo*, though usually the curvature was abnormally pronounced *in vitro*. In this group, however, some of the radii and ulnae failed to develop anatomically or to ossify and merely enlarged to form an oval nodule of small-celled cartilage; most of the radii and ulnae had fairly well-shaped proximal ends, but owing to the difficulty of separating them cleanly from the carpus, the distal ends were either imperfect or associated with carpal fragments. The femur formed its pulley-like condyles, a head and sometimes a trochanter, though the primordium of the trochanter was often destroyed during dissection. In the explanted tibiae, the general outline and relative sizes of the ends were fairly normal. This capacity of the rudiments for self-differentiation has been discussed elsewhere (Fell, 1939, 1952).

During the first 2 days *in vitro*, the development of the rudiments grown in medium containing 16 μg/100 ml. of thyroxine resembled that of the controls, but by the 4th day the shaft of the femur, and often of the tibia also, was seen to be shorter than in the corresponding explants in control medium; the ends

were unaffected. With more prolonged cultivation, this difference between the T-treated and control rudiments increased in the tibiae and femora, and by the 6th day had also appeared in the humeri. On the other hand, there was no significant difference in shape between the T-treated and control radii and ulnae throughout the period of cultivation.

Histologically, the explants attained an advanced stage of differentiation. Controls fixed after 4 days' cultivation showed a well developed region of hypertrophic cartilage and fairly broad zones of flattened cells which were already demarcated from the small-celled epiphyses; a periosteum enveloped

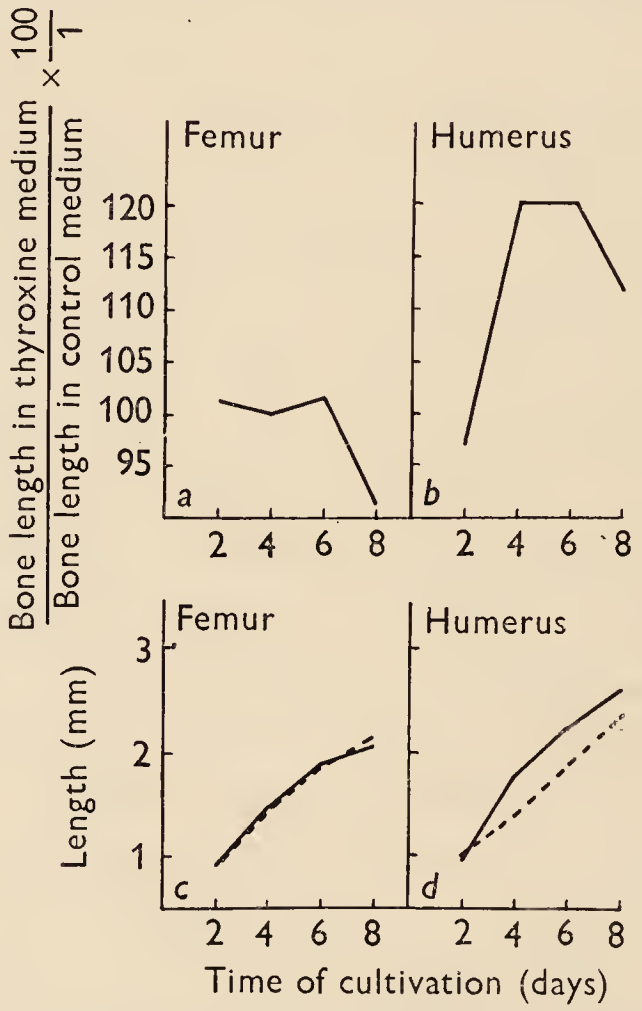


Text-fig. 4. Camera lucida drawings of the living long-bones from the legs and wings of an embryo of group III. One of each pair was grown for 8 days in medium containing 16 μg thyroxine/100 ml. and the other in control medium. Note the differential effect of the hormone on the growth of the various rudiments; as compared with the controls, the shaft is much shorter in the T-treated humerus, femur and tibia, slightly shorter in the treated ulna, but longer in the T-treated radius.

the shaft on which a thin layer of bone had been laid. In control medium (Pl. 1, figs. 3, 5), the density of the cartilaginous matrix rapidly increased and in the largest explants, especially in the tibiae, the chondroblasts in the middle segment, where hypertrophy had first appeared, were somewhat shrunken by the 8th day; in the embryo this part of the shaft would have been excavated at this stage and replaced by marrow, but in chick rudiments this does not happen *in vitro*. In the distal parts of the shaft, the hypertrophic cartilage was highly developed and the zones of flattened cells were now sharply bounded

from the epiphyses. The periosteal bone, though still a simple layer with only slight indication of a trabecular structure, was much thicker.

The histological effects of thyroxine are summarized in Table 2. It will be seen that, as in the explanted blastemata, the different rudiments did not react to the hormone in the same way. All the humeri fixed after 4–6 days were better differentiated in the presence of thyroxine than in control medium; after 7–10 days, however, the development of the controls had often caught up with that of the T-treated explants, and the degree of differentiation was



Text-fig. 5. Groups I and II: (*a*) and (*b*) represent the relative rate of growth of embryonic bones (femur and humerus) on thyroxine medium compared with that of the corresponding rudiments on control medium. Ordinates represent $\frac{\text{bone length on thyroxine medium}}{\text{bone length on control medium}} \times 100$: abscissae represent days of culture. (*c*) and (*d*) are average growth curves of embryonic femora and humeri on control medium (dotted lines) as compared with that of the corresponding bones on thyroxine medium (plain lines). Note the stimulation of the growth of the humerus during the first 6 days. During this period there was no increased growth of the femur. After the 6th day both femora and humeri on the thyroxine medium grew less well than the controls.

either the same in both (6 pairs) or only slightly more advanced in the T-treated rudiments (8 pairs). In several of the humeri fixed at this stage, some of the hypertrophic cells were undergoing the characteristic type of degeneration described in the previous section and, especially at the periphery, the intercellular partitions were much thinner than in the controls; these changes were probably partly responsible for the diminished growth of the shaft seen in the living cultures from the 6th day onwards. Four pairs of radii

(group III) failed to undergo chondroblastic hypertrophy in either medium; and in one pair hypertrophy appeared in the T-treated rudiment only. In all of the remaining 13 pairs hypertrophy was more advanced in the T-treated than in the control rudiments. In 3 pairs of ulnae also the cells failed to hypertrophy and in 1 pair they enlarged only in the T-treated rudiment; in 12 of the other 15 pairs the thyroxine explants were the more advanced (Pl. 1, figs. 7, 8) and in the rest there was no difference. Regressive changes were seen in only 2 of the T-treated ulnae.

TABLE 2. Groups III and IV

Culture period	Rudiment	Total no. of pairs	Degree of maturation of cartilage in pairs of rudiments: Greater in *T*	Same in both	Greater in *C*	Absent from both
4–6 days	Humerus	5	5	—	—	—
	Radius	4	2	—	—	2
	Ulna	5	3	—	—	2
	Femur	5	1	2	2	—
	Tibia	5	3	2	—	—
7–10 days	Humerus	14	8	6	—	—
	Radius	14	12	—	—	2
	Ulna	14	10	3	—	1
	Femur	8	—	7	1	—
	Tibia	8	1	7	—	—

The degree of maturation (chondroblastic hypertrophy) in paired explants of isolated bone rudiments from $5\frac{1}{2}$ to $6\frac{1}{2}$-day embryos. *T* = medium + thyroxine 16 μg/100 ml.; *C* = control medium. For definition of groups see p. 429. One of each pair from the same chick was grown in *T* and the other in *C*. *N.B.* All the radii and ulnae showing no chondroblastic hypertrophy belonged to group III.

The femur and tibia were more severely affected than the rudiments of the wing-bones. As described above, in the T-treated humeri the intercellular partitions in the hypertrophic zone, especially near the periphery, were sometimes much thinner than in the controls; this effect was often very pronounced in the femora and tibiae, in which the peripheral chondroblasts appeared crowded together and were often separated by a mere film of matrix (Pl. 1, figs. 5, 6). In normal development hypertrophy spreads gradually from the interior of the shaft towards the surface, but under the influence of thyroxine this process appeared to be accelerated so that the peripheral chondroblasts enlarged prematurely, which reduced the production of matrix in this region. In the femur and tibia this effect was particularly noticeable at about the 6th or 7th day; after this stage the difference between the treated and untreated rudiments became less obvious, as the peripheral chondroblasts of the controls had also enlarged while the intercellular partitions of the experimental explants had thickened somewhat, though remaining considerably thinner than those of the controls. The proliferative zones of flattened cells in the T-treated femora and tibiae were often much narrower than those of the

control explants; whether this was owing to a diminished mitotic rate, to the premature hypertrophy of the flattened cells or to both these factors is not known, but this reduction of the proliferative zone was also partly responsible for the retarded growth of the diaphysis in the presence of the hormone. Although hypertrophy seemed to be accelerated at the periphery of the shaft in the femur and tibia, the chondroblasts, unlike those of the wing rudiments, sometimes did not enlarge as much as those of the controls and usually many were degenerate.

In all the rudiments, including the femur and tibia, the periosteal bone was usually as healthy and well developed as in the controls and sometimes more plentiful (Pl. 1, figs. 5, 6).

To summarize the results of these experiments: in the humeri, chondroblastic hypertrophy was at first stimulated, but later the rudiments sometimes underwent characteristic regressive changes; the radii were either stimulated or unaffected and so also were the ulnae, although occasionally regressive changes appeared towards the end of the culture period; the femur and tibia were more adversely affected by the hormone than the wing-bone rudiments but seemed to show some acceleration of differentiation at the periphery of the shaft.

One set of rudiments was grown in a higher concentration of thyroxine, viz. 400 μg/100 ml. In the living cultures the treated femur attained only about two-thirds the length of its control and the condylar end was bent at an angle to the shaft; the T-treated tibia and humerus also were much shorter than those in control medium, but the length of the T-treated ulna was only slightly less than that of its control, and the T-treated radius was considerably longer than that in control medium. The explants were fixed after 6 days' cultivation. The histological changes produced by thyroxine in the femur, tibia, humerus and ulna were similar to those described above for the lower concentration, but more drastic. Many of the hypertrophic cells in the femur and tibia and some of those in the humerus and ulna were degenerate and, especially in the two leg-bones, the amount of intercellular material between the hypertrophic chondroblasts was much reduced, though its metachromasia was normal. In the T-treated femur and tibia the zones of flattened cells had almost disappeared and they were much reduced in the humerus and ulna. The radius grown in the presence of the hormone was healthy and better developed than its control. Periosteal ossification was about the same in both sets of explants, but in the thyroxine medium many of the osteoblasts of the femur were degenerate.

Growth. It will be seen from Text-fig. 1 that the rates of growth in the living embryo are much greater than those of the corresponding bones grown in control medium *in vitro*. In general, 8 days' growth in tissue culture gives a length of bone produced in the living chick in times varying from 2 to 3 days.

The average rates of growth in Text-fig. 6 are composite and are based on measurements of bones of groups III and IV cultivated both at the Strangeways Laboratory and at Mill Hill. Seventeen pairs of femora, 25 pairs of tibiae, 27 pairs of humeri, 28 pairs of radii and 13 pairs of ulnae were so

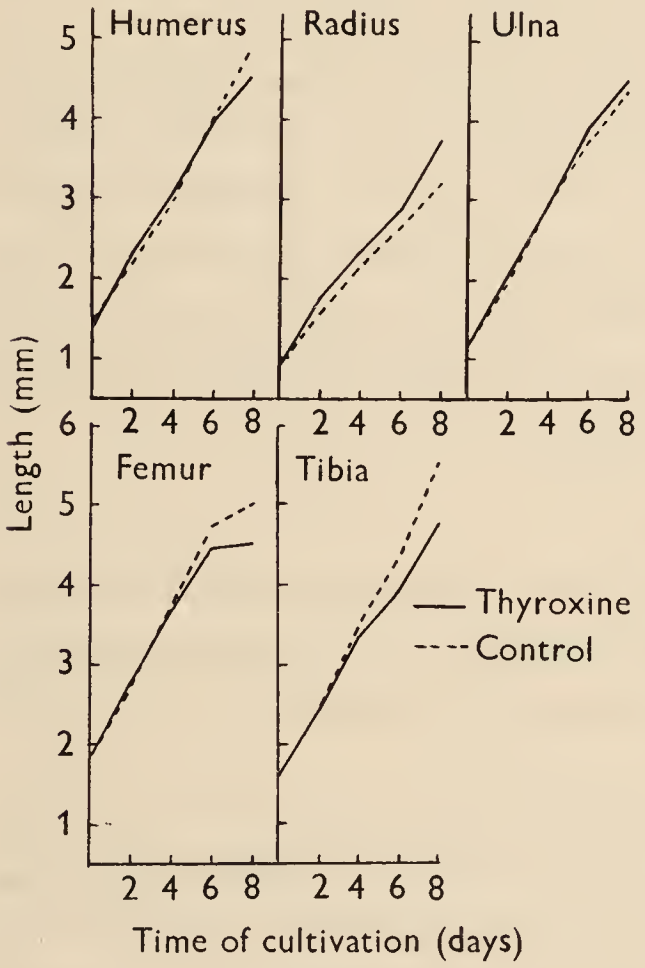


Fig. 6.

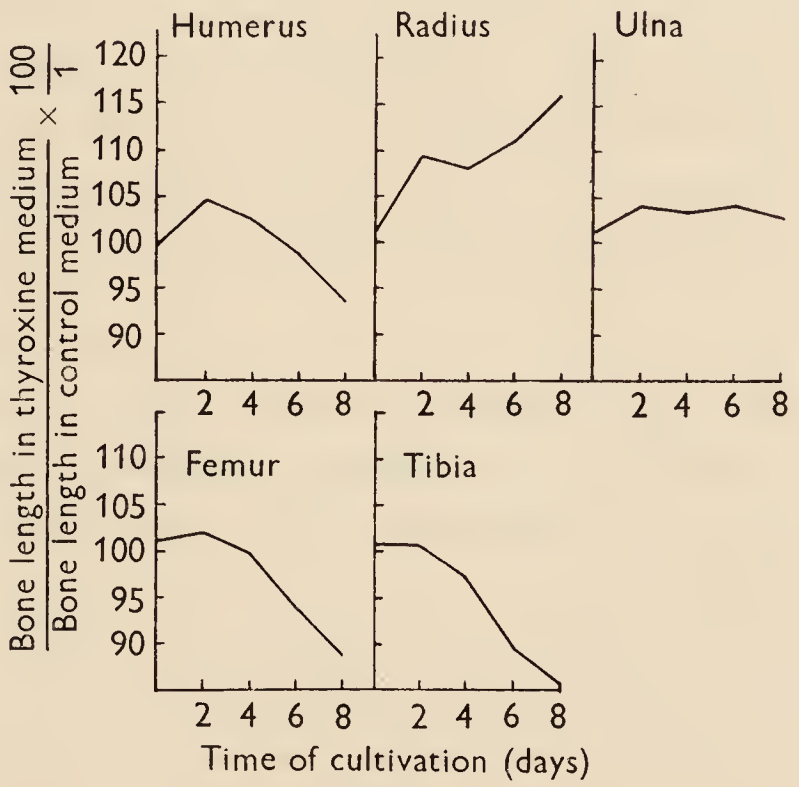


Fig. 7.

Text-fig. 6. Groups III and IV: the average growth of embryonic bones on control medium (dotted lines) as compared with that of the corresponding bones on thyroxine medium (plain lines). (16 μg L-thyroxine added per 100 ml. of medium.) Ordinates represent length of bones in mm; abscissae represent days of culture. Note the stimulating effect of the thyroxine on the growth of the radius. From the 6th day after explantation the growth is usually depressed by the thyroxine except in the case of the radius and ulna.

Text-fig. 7. Groups III and IV: the average relative rate of growth of embryonic bones on thyroxine medium (16 μg L-thyroxine added per 100 ml. of medium) compared with that of the corresponding bones on control medium. Ordinates represent

$$\frac{\text{bone length on thyroxine medium}}{\text{bone length on control medium}} \times 100;$$

abscissae represent days of culture. Note the stimulation of growth of the radius. In the ulna, femur and tibia, the early stimulation of growth is small. During the later days all the bones on the thyroxine medium except the radius and ulna show a reduced rate of growth.

measured. Text-fig. 7 records the average lengths of the T-treated bones as percentages of the lengths of the controls of groups III and IV on certain days during the experiment. Both series of graphs show that the growth of the leg-bones (femur and tibia) was seriously retarded by the thyroxine after the first 2–4 days of incubation (statistically significant); even in the first 2–4 days the femur and tibia in these groups showed hardly any greater increase in

length under the influence of thyroxine. On the other hand, the average growth rate of the T-treated humeri was slightly, but significantly, above that of the controls for the first 2 days *in vitro* and then fell significantly below the control value between the 6th and 8th days. The T-treated radii grew more rapidly during the whole period of observation, but the increased growth was only significant during the 0–2 days and the 6–8 days intervals.

It is clear that the effect of thyroxine on the growth of the different rudiments of the same embryonic age varied greatly, the radii and humeri responding best to the stimulating effect of thyroxine as judged both by growth rate and chondrogenesis, although the increased growth was not maintained in the case of the humerus. The femora and tibiae were most susceptible to the harmful effect of thyroxine as measured by growth and showed only slight acceleration of chondrogenesis.

DISCUSSION

The object of the experiments described above was to determine the biological rather than the toxicological effects of thyroxine. It was therefore necessary to use concentrations of the hormone of the same order as those present in the living animal.

The amount of thyroid active principle in fowl blood is not known, and the best available guide is probably the concentration said to be present in human blood. These estimates were made before the discovery by Gross & Pitt-Rivers (1952) of triiodothyronine as a thyroid constituent—a substance with greater biological activity than thyroxine—but the iodine content of this substance would be included in the following figures. It is evident that the actual human figures can only be a rough indication of the concentrations of these active principles in fowl plasma. Means (1948), quoting data supplied by D. S. Riggs, says: 'The serum protein-bound iodine of euthyroid individuals normally falls in the range of 3·5–7 μg./100 ml. (gamma per cent.). In hyperthyroidism it is above 8 μg. per cent. and may be elevated to 20 or 30 μg. per cent., although values of from 10 to 16 μg. per cent. are more usual. In hypothyroidism, values below 3·0 μg. per cent. are characteristic; in outspoken myxoedema the serum-precipitable iodine is commonly less than 1·0 μg. per cent.'

When these values for iodine are converted to those for thyroxine the following may be regarded as the approximate total iodine-containing active principle of human serum calculated as thyroxine: normal, 5–10 μg/100 ml.; hyperthyroidism, 12–45 μg/100 ml.; myxoedema, 1·5–4·5 μg/100 ml.

It has been pointed out that the X_2 medium of the present work contains an added 16 μg/100 ml. of thyroxine and medium X_6 an additional 1 μg/100 ml. Since the L-thyroxine was added to normal birds' plasma which must have contained some thyroxine, on the assumption that this amount and that of the embryo extract are of the order of that present in human serum, the total

thyroxine content of X_2 medium must have been about 21–26 μg/100 ml., which would raise it to a concentration of human hyperthyroidic serum. The X_6 plasma, on the other hand, only had an additional 1 μg/100 ml., which would increase its total amount to 6–11 μg/100 ml. and thus leave the plasma equivalent to that of a normal individual. It would appear, therefore, that the thyroxine content of the culture media fell within the limits normally met with in biological conditions and was not exceptionally high.

The results have shown that under these conditions thyroxine has a direct action on skeletal tissue *in vitro*, where all systemic effects are eliminated. The same concentration of the added hormone, e.g. 16 μg/100 ml., may be ineffective, stimulatory or toxic according to the stage of differentiation of the tissue at the beginning of treatment and the particular bone rudiment exposed.

We will consider first the stimulatory effect. In general, the young rudiments were more susceptible to stimulation by thyroxine than the older primordia and, as recorded in Table 1, the blastemata were affected even by the addition of a concentration as low as 1 μg/100 ml. (X_6). Of all the rudiments, the humerus responded best and differentiation was accelerated, not only in those explanted at the blastematous stage, but also in the older rudiments during the first few days of cultivation. Of the other wing-bones, the radii were occasionally stimulated in the blastemata and in all the older explants; the ulna was not affected in the blastemata but was often stimulated in groups III and IV. In the tibiae and femora maturation of the cartilage was sometimes hastened in the blastemata but only very slightly at the older stage.

The older explants were more liable to toxic action than the blastemata, but here again the various rudiments responded differently to the same dose. Thus the leg-bones were always more severely affected than those of the wing. Of the wing-bones, the humerus was the most susceptible and next the ulna; the radius was the most resistant, and even when exposed to a concentration of 400 μg/100 ml., which produced regressive changes in all the other rudiments, it remained healthy during the period of the experiment and was more highly differentiated than its control.

As stated above, Willier (1924) grafted fragments of thyroid gland on to the chorio-allantoic membranes of chick embryos *in ovo*, and noted emaciation of the entire chick and shortening of the limbs. Although the author gives no histological data, his results appear to agree with ours in that the leg-bones of the host were more affected by the graft than the skeleton: 'when the host embryo is only slightly affected by the thyroid graft, the modifications show up most clearly in the legs'.

On the other hand, when Beyer (1952) injected thyroxine into unincubated eggs, he found that the development and growth of the embryos were accelerated. It seems probable that the disparity between Willier's and Beyer's results is due to the fact that the former produced hyperthyroidism

during the later stages of development when, according to our observations, the hormone has an inhibitory effect on skeletal growth, while the latter subjected the embryo to the action of thyroxine at an early stage when our results suggest that the tissue is most susceptible to stimulation of development. Beyer states that the increase in weight of the treated chicks over that of the controls was greatest at 6 days and then diminished to hatching time.

The variation in the response of different bone-rudiments to the same agent is an interesting phenomenon which was also encountered in earlier experiments on the effects of abnormally high concentrations of vitamin A on the explanted limb-bone primordia of 6-day chick embryos. Excess vitamin A affected the rudiments in the same order of severity as thyroxine, i.e. the most susceptible to damage was the femur, followed by the tibia, humerus, ulna and radius respectively. The histological effects of the two agents, however, were very different. Thus vitamin A did not accelerate differentiation in very young skeletal rudiments as did thyroxine; instead, either chondrogenesis was inhibited or else early cartilage developed but rapidly disintegrated owing to dissolution of the matrix (Fell & Mellanby, unpublished). In older 6-day rudiments, excess vitamin A caused the matrix to lose its characteristic metachromasia, while thyroxine did not alter the normal staining reaction of the intercellular material. Both agents reduced the production of matrix but this reduction was much greater with the vitamin than with the hormone.

Why there should be this differential response of the long-bone rudiments to the same agent is not known. As described above, in normal embryonic development, the primordia of the long-bones differ from one another in both their histological development and growth-rate, but what physiological characteristics determine their response to thyroxine or vitamin A remain obscure; we can only say that the two rudiments which differentiate most slowly *in vivo*, viz. the ulna and radius, are the most resistant *in vitro* to the injurious action of the two agents.

How far the effects of thyroxine on embryonic skeletal rudiments in culture are comparable with the clinical effects of hyperthyroidism on the post-embryonic skeleton is doubtful. The clinical results show that, directly or indirectly, the hormone acts on bones in two ways.

(1) It is concerned in normal bone growth, so that when it is deficient growth and development are greatly retarded.

(2) Excessive amounts cause osteoporosis which is said to be due to exaggerated osteoclasis without arrest of the calcifying process.

The first of these two actions is physiological, and perhaps may be related to the accelerated maturation of cartilage seen in the present study. The second effect is pathological, is manifested in well-developed bone which is very different from the early embryonic material used in our experiments and probably has no counterpart in our results.

On the other hand, some of the effects obtained experimentally by the administration of excess thyroxine to young animals, are very similar to those produced in the explants *in vitro*. Thus the acceleration of the developmental changes of growing bones and their premature ageing in treated animals, referred to in the introduction (Dott, 1923; Silberberg & Silberberg, 1938, 1940; Simpson *et al.* 1950), appear to be essentially the same phenomenon as that seen in the embryonic bones in culture.

SUMMARY

1. Chick long-bone rudiments cultivated *in vitro* were affected by L-thyroxine added to the culture medium in amounts which fell within the limits encountered naturally in the blood plasma in human hyperthyroid conditions.

2. Two types of reaction were found: (*a*) a stimulatory effect on the maturation of cartilage, and (*b*) a toxic effect causing retardation of growth and cellular degeneration.

3. The stimulatory effect of L-thyroxine, as seen histologically in the accelerated maturation of the cartilage, was more pronounced in rudiments explanted at the blastematous stage except in the case of the ulna, but also appeared in some of the older bone primordia during the first few days of cultivation. Different bones were stimulated to different degrees by the same concentrations of L-thyroxine. At the blastematous stage, the humerus responded best; in the older group the response was best in the humerus, radius and ulna and least in the tibia and femur.

4. The toxic effect was most conspicuous in the older rudiments and during the later stages of cultivation. The leg-bones were more severely affected than those of the wing, the femur being slightly more damaged than the tibia. Of the wing-bones, the humerus was the most susceptible and the radius the most resistant.

5. These experiments show that L-thyroxine acts directly on the skeletal rudiments when all systemic effects are eliminated.

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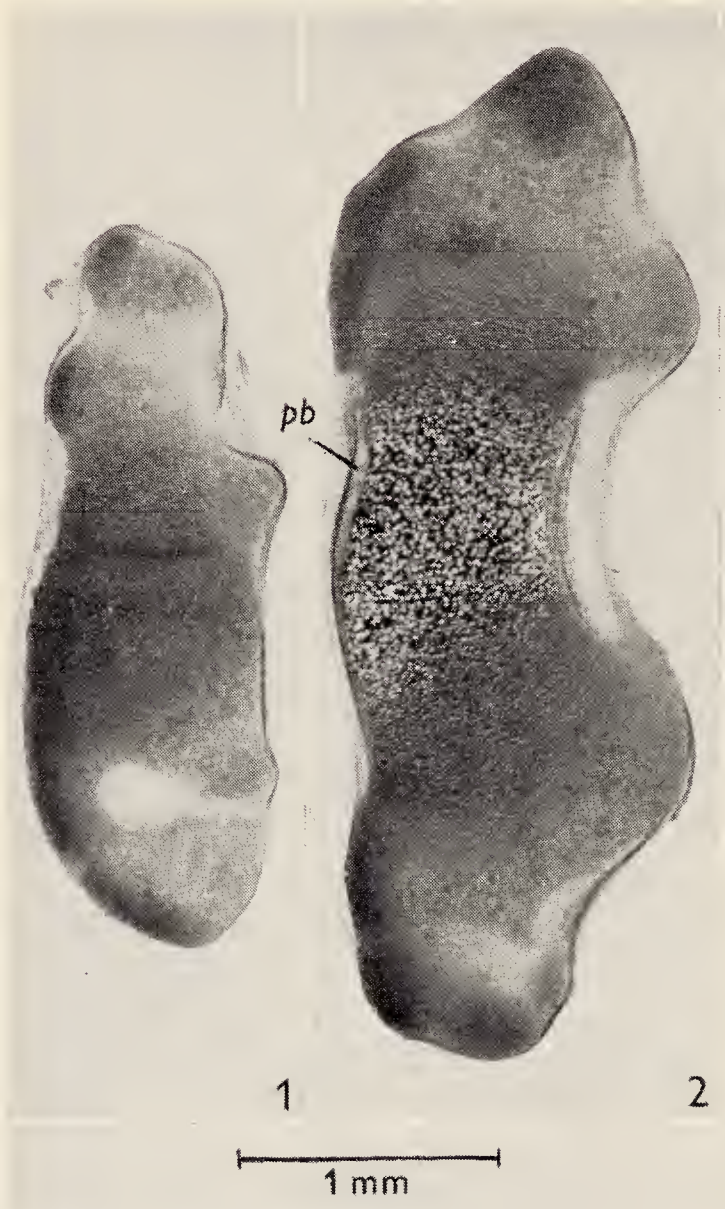

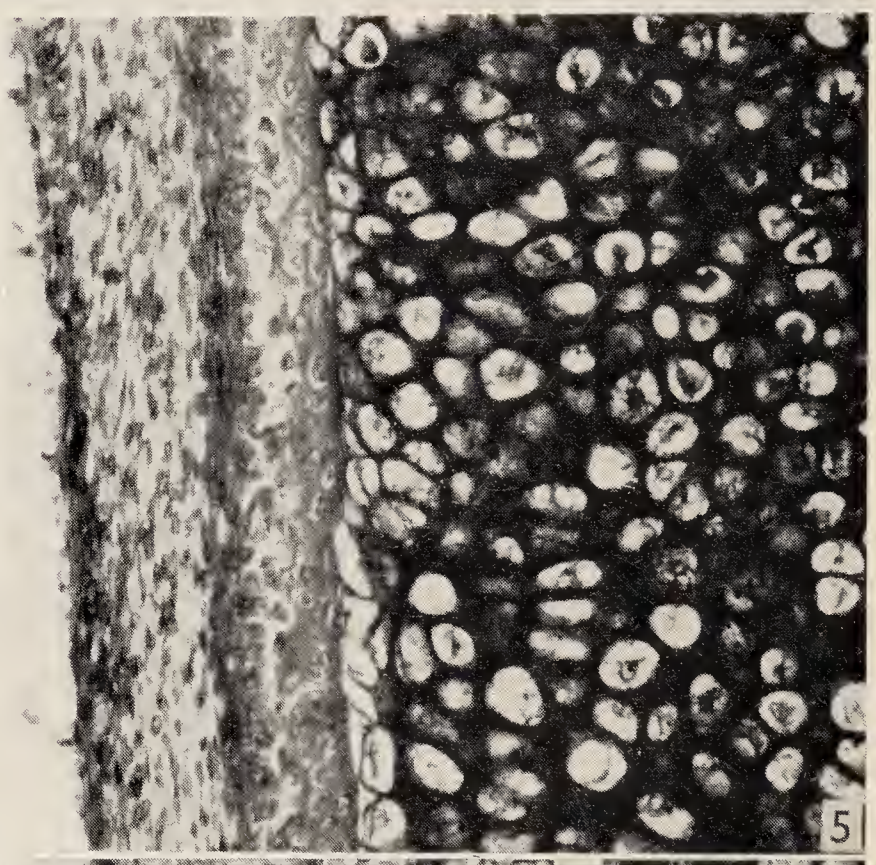

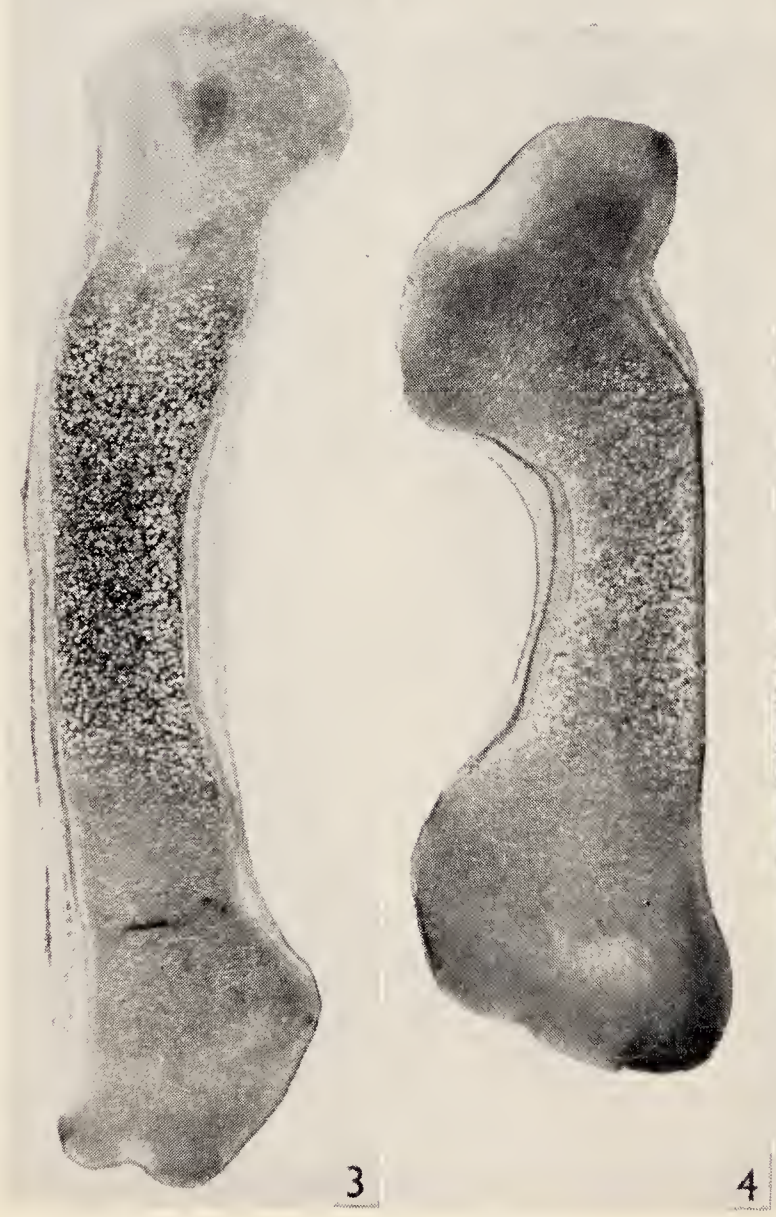

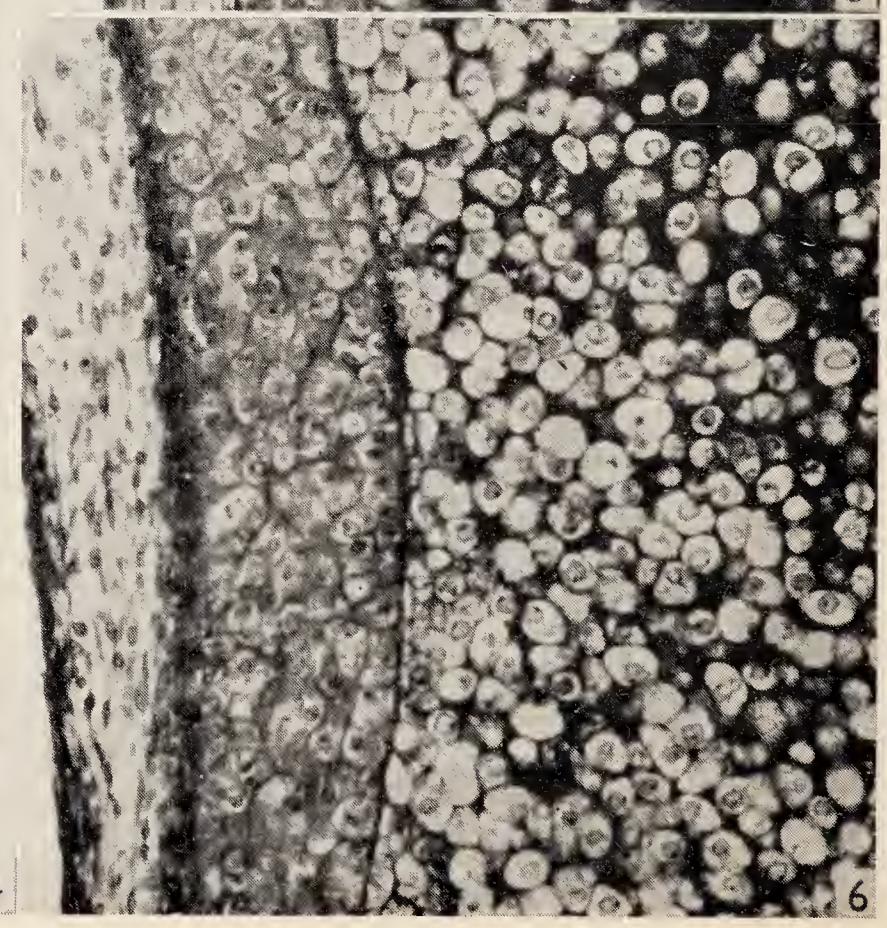

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EXPLANATION OF PLATE

(Photographs by Mr V. C. Norfield, Strangeways Research Laboratory)

All the sections were stained with Delafield's haematoxylin and chromotrop. The magnification of figs. 1–4, 7 and 8 is indicated by the scale on the left, that of figs. 5 and 6 by the right-hand scale.

Fig. 1. A control skeletal blastema from the wing-bud of an embryo of group I, after 9 days' cultivation in control medium. The humerus shows no chondroblastic hypertrophy or ossification.

Fig. 2. The opposite wing-blastema from the same chick after 9 days' growth in medium containing 16 μg/100 ml. of added L-thyroxine. The development of the explant has been stimulated; it is larger than its control, in the shaft of the humerus the cartilage cells have undergone hypertrophy and periosteal ossification has begun.

Fig. 3. A control tibia from an embryo of group III after 6 days *in vitro*; the cartilage cells of the shaft have hypertrophied and periosteal bone has been formed.

Fig. 4. The opposite tibia from the same embryo as the control shown in fig. 3, after 6 days in medium containing 16 μg/100 ml. of added L-thyroxine. The hormone has affected the tibia adversely; the shaft is much shorter than in the control, but shows chondroblastic hypertrophy and periosteal ossification.

Fig. 5. Cartilage and periosteal bone in the same section as that shown in fig. 3; after 6 days in control medium the hypertrophic chondroblasts are separated by broad partitions of matrix.

Fig. 6. Cartilage and periosteal bone in the same section as that shown in fig. 4; after 6 days in medium containing added thyroxine, only narrow partitions of cartilaginous matrix have been formed and the hypertrophic cells are crowded together.

Fig. 7. Control ulna from the same embryo as the tibiae shown in figs. 3–6; 6 days in control culture.

Fig. 8. Opposite ulna from the same embryo as the rudiment shown in fig. 7, after 6 days in medium containing 16 μg/100 ml. of added L-thyroxine. The ulna is less susceptible to damage by the hormone than the tibia; the hypertrophic cartilage is rather more advanced than in the control, but the explant is otherwise unaffected.